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# Egg Wash Wastewater: Estrogenic Risk or Environmental Asset?

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## ABSTRACT

Commercial production of eggs and egg products requires the washing of eggs to remove urinary-fecal material and broken egg residue. In the case of one Ohio farming facility, 1.6 million birds produce 1.4 million eggs per day, using approximately 50 mL of wash water/egg or approximately 70 000 L per day. The aqueous waste stream was evaluated for estrogenicity to determine if potential for endocrine disruption would result from agricultural application of such wastewater. Samples collected the Fall (October) of 2010 included: water from 2 egg washers operating in series, inlet pipe to the treatment lagoon, a lagoon composite, and products used within the facility in the cleaning of equipment and treatment of the waste. In February 2011, the treatment lagoon was fitted with an extensive aeration system and subsequent sample sets were collected on 3 consecutive days in May and November. Samples were extracted by solid phase extraction and assayed for estrogenic activity using the in vitro E-Screen assay. Raw untreated wastewater from the egg washers contained 17β-estradiol equivalents ($E_2$Eqs) ranging from 9 to 18 ng/L, pipe grab samples entering into the treatment lagoon ranged from <0.14 to 4.4 ng/L (variability related to time of emptying of egg wash tanks), whereas treatment lagoon water contained 0.3 to 4.0 ng/L $E_2$Eq. Addition of an aeration system to the treatment lagoon eliminated surface "frothing," reduced noxious odor emission, and $E_2$Eqs were lower than the pre-aeration concentrations (4 ng/L [$n = 1$, no statistical comparison possible] vs 0.3 to 1.4 ng/L in 2011). Because of matrix effects, estrogens were not quantifiable by LC-MS2 in even egg washwater extracts, at concentrations in which internal deuterated estrogen standards were quantifiable. Estrone and $E_2$ parent ions were detected in egg washwater samples only, and confirmatory ion fragments were detected in only one of these samples. Estrogenicity of the wastewater from the treatment lagoon was already at the proposed aquatic no effect concentration for 17β-E2 and would be expected to decrease further as wastewater passes through 2 consecutive storage ponds before application on field crops for irrigation. The original project plan was to follow the wastewater as it was applied by aerial irrigation and concomitant surface runoff, but based on the consistent and extremely low concentration of estrogenic activity of the wastewater from the treatment lagoon, it was concluded that activity would be below limits of quantitation by E-Screen in water used for irrigation from the storage ponds. Use of egg wash wastewater—or gray water—to irrigate crops removes the cost and burden of wastewater treatment by the local wastewater plant, poses little to no potential threat of estrogenic endocrine disruption, and supports the conservation of water resources through the use of wastewater irrigation. *Integr Environ Assess Manag* 2013;9:517–523. © 2013 SETAC

**Keywords:** Egg wash E-Screen Lagoon MCF-7 Wastewaters



## INTRODUCTION

Many agricultural facilities have been investigated in attempts to determine potential for release of endocrine disruptors into the aquatic environment, especially estrogenic compounds originating from confined animal feeding operations. Avian species have been reported to have higher levels of circulating estradiol (≈380 pg/mL or $1.4 \times 10^{-9}$ M) (Cockrem and Rounce 1994) than mammals, including humans (≈30–200 pg/mL or $0.1–0.7 \times 10^{-9}$ M) (Baird and Guevara 1969; Munro et al. 1991). Commercial production of eggs and egg products requires the washing of eggs to remove urinary-fecal material and broken egg residue. In the case of one Ohio farming facility, 1.6 million birds produce 1.4 million eggs per day resulting in approximately 47 000 L of raw egg product that is stored in refrigerated silos (Figure 1A). Approximately 50 mL of rinse water is required per egg or approximately 70 000 L for a typical production day. The goal was to investigate the impact of surface application of litter and egg washwater on local surface waters surrounding such a large egg production facility. The original project plan was to measure estrogenicity of wastewater through the facility, test all chemicals being added to the waste stream (cleaners, disinfectants, antifoaming agents), evaluate treatment and storage lagoon waters for estrogenicity, and to determine the terminal estrogenic concentrations as processed water was applied by aerial irrigation and concomitant surface runoff. In a similar manner, the composted excrement collected from caged birds would be evaluated for total estrogenicity and then followed in surface runoff over time after surface application to agricultural fields. No opportunity to study runoff postapplication of poultry manure existed, as all of the composted excrement from the

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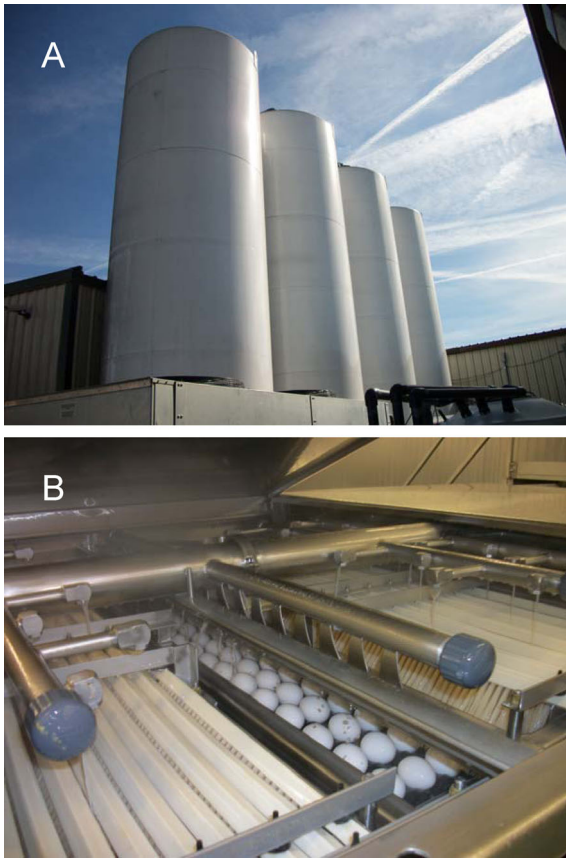


**Figure 1.** (**A**) Refrigerated silos where egg product is stored before pickup by refrigerated tankers. Individual silo capacity is approximately 45 000 L, and daily production fills approximately 1.2 to 1.5 silos. (**B**) Top view of egg washer. Manure and broken egg residue was removed from eggs by sprays with Chloroclean 269 (a chlorinated alkaline cleanser used in 2010) or with Apex 292 (another alkaline product that precipitates proteins). The pH of washwater was monitored, and product added to maintain a pH of 9.5–10. Although the 2 washers operate in series, an overflow system in Washer 2 allows for recirculation of washwater back to Washer 1 to maintain adequate washwater levels. Washers are dumped and cleaned after a maximum of 4 h, typically having processed 56 cases of eggs (≈20 000 eggs).

layer hens is sold and transported off-site as fertilizer, with some of the pelletized form even used on the US White House lawns.

The E-Screen, an in vitro assay originally developed to evaluate the estrogenicity of pure chemicals (Soto et al. 1995), was used to determine estrogenic activity of the egg washwater waste stream as it progressed through the facility and into the treatment lagoon. The sampling schedule was designed to evaluate day-to-day as well as seasonal variation of the waste stream.

## MATERIALS AND METHODS

### *Site description and experimental design*

An initial set of samples were collected in October 2010 to determine feasibility of estrogen extraction, appropriate sample volumes, potential toxicity of chemicals in use on E-Screen cells, and adjustment of sampling scheme, based on findings. Egg washers are connected in series (2), so that water from one tank is commingled with water from the other (Figure 1B). When eggs are transferred from Washer 1 to Washer 2, water is lost from Washer 1, and the water is replaced through an overflow system from Washer 2, maintaining a balance in water levels between the 2 tanks. Both tanks are emptied and cleaned after a maximum 4-hour cycle. An initial sample set was collected from egg Washer 1 (3 h of operation, 432 000 eggs), Washer 2 (1 h and 4 h, 144 000 eggs and 576 000 eggs, respectively), the inlet pipe to the treatment lagoon (2 samples ≈3 h apart), and the treatment lagoon surface (composite) (Sample list, Table 1). At the time of the 2010 sampling, the treatment lagoon was anaerobic (Figure 2A) with an 11.4 million L capacity, and a residence time of up to 90 days (seasonal lack of irrigation required longer storage time, 30-day minimum residence time). The treatment lagoon cascaded into 2 sequential holding ponds (earthen clay-lined, $9.2 \times 10^6$ L capacity in the first, and $2.0 \times 10^6$ L in the second) before aerial application for irrigation purposes from the terminal pond (Figure 2C and D).

Between the initial sample collection in October of 2010 and May of 2011, the second storage pond was drained, dredged, and fitted with an extensive aeration system at a cost of $250 000 to function as the 1° treatment lagoon (Figures 2B and 3, schematic). The aeration system consisted of tubing running across the lagoon in 5 parallel lines that floated on the surface. The tubing was fitted with perpendicular lines equipped with terminal diffusers that supplied air to the bottom of the lagoon (2 m deep) at a rate of 28.3 $m^3$ of air/min per line. The system was completed in February of 2011.

Chemicals in use at the facility at the time of sampling were also tested for estrogenicity and toxicity. These chemicals obtained from Hydrite Chemical (Brookfield, WI) were Chloroclean 269 (chlorinated alkaline cleanser used on eggs), Alkali LF 257 (equipment cleaning agent), Dura-Foam 263 (chlorinated foam cleaner), and Suppressor 3110 (defoamer); all of which were replaced in 2011 by Apex 292 (an alkaline product that precipitates proteins). Samples (≈115 mL in high

**Table 1.** Sample collections

| Season–date | Sample type or chemicals for cleaning |
|---|---|
| Preliminary samples<br>Fall 2010<br>10/24/10 | Washwater 1 (wash time = 3 h)<br>Washwater 2 (wash time = 1 and 4 h)<br>Pipe grab (flowing into treatment lagoon) (1 h apart) [a]<br>Treatment lagoon composite<br>Alkali 257<br>Durafoam 263<br>Chlorclean 296<br>Suppressor 3100 |
| Spring 2011 [b]<br>5/16/11–5/18/11 | Washwater 1 (wash time = 3 h)<br>Washwater 2 (wash time = 3 h)<br>Pipe grab (flowing into treatment lagoon)<br>Treatment lagoon composite<br>Apex 292 |
| Fall 2011 [b]<br>11/8/11–11/10/11 | Washwater 1 (wash time = 3 h)<br>Washwater 1 (wash time = 3 h)<br>Treatment lagoon composite |

[a]Liquids from egg washwater, tanker truck, and silo washouts flow into holding tanks that are connected in series. The second tank is pumped down and into the lagoon when it reaches its maximum holding capacity. The output of this pipe is not a mirror reflection of what was in the egg washers, which are dumped and washed out after a maximum of 4 h of use.
[b]Samples were collected at the same time each day.


**Figure 2.** (**A**) Anaerobic treatment lagoon in operation at the time of 2010 sampling. Egg wash wastewater and water from cleaning of washing units is pumped into the treatment lagoon. Flocculent material seen in photograph often formed on the surface. Composite samples were collected from each side of the lagoon. (**B**) Aerobic treatment lagoon, postinstallation of aeration system (2011). Air lines are suspended perpendicular to the parallel pipes floating on the lagoon surface, and terminate with diffuser nozzles through which air is delivered to the bottom of the lagoon (2 m depth) at a rate of 28.3 $m^3$ of air/min per line. (**C**) Storage ponds. Wastewater cascades from the treatment lagoon into the pond in the foreground, and subsequently into the last storage pond seen in the background. (**D**) Aerial pivot irrigation system where wastewater from terminal storage pond is applied to corn crop, in rotation, once every 3 years, when soil conditions allow as per Ohio Environmental Protection Agency regulations.

density polyethylene bottles) were collected, frozen at −20° C and shipped on ice to Fargo, ND for later extraction and E-Screen analysis.

To assess daily and spring versus fall variation of waste stream estrogenicity, samples sets were collected on 3 consecutive days in Spring (May) and Fall (November) of 2011 (Table 1). Egg washwater was collected from Washer 1 and 2 after 3 to 4 h of operation (numbers of eggs processed were similar to those cited above), and a composite treatment lagoon sample at the same time of day. The cleaner/chemical in use during these 2 sampling periods was APEX 292 (Hydrite Chemical). Sampling and shipping were as described above.

### *Glassware and reagents*

All glassware used for sample extraction or analysis of estrogenicity was solvent washed and baked. The process was: wash in Liquinox detergent (MG Scientific, Pleasant Prairie, WI), rinse with nanopure water (np$H_2O$), dry, rinse with series of solvents (acetone, methanol, acetonitrile, ethyl acetate, and methylene chloride), and bake at 450° C for 4 h. All solvents used were high performance liquid chromatography grade (99.8% purity). Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

### *Sample extraction*

Wastewater from egg washers, pipe grabs, and lagoon composite samples were thawed, shaken, and filtered through glass wool (previously washed with solvents: acetone, methanol, acetonitrile). Filtering was necessary to remove solids (primarily coagulated egg material typically removed from the waste stream and sold as a protein amendment for use in pet food). Filtrate was concentrated by solid phase extraction (SPE, OASIS HLB, 200 mg, 5 mL; Waters, Milford, MA) and taken to dryness exactly as described by Shappell et al. (2012). Extraction volumes were 110 mL for October 2010 samples, and 250 mL for all subsequent samples. Dry eluates were stored at −20° C for later analysis.

### *E-Screen analysis*

The MCF-7 BOS, estrogen-dependent cell line (derived from a human mammary epithelial carcinoma) was used to evaluate samples for estrogenicity relative to 17β-estradiol (17β-$E_2$) as previously described (Shappell 2006). Dry SPE eluates were resuspended in np$H_2O$ (110 mL samples to 70.4 μL, and 250 mL samples into 160 μL), and further diluted in steroid-free medium (no phenol red, and 10% charcoal

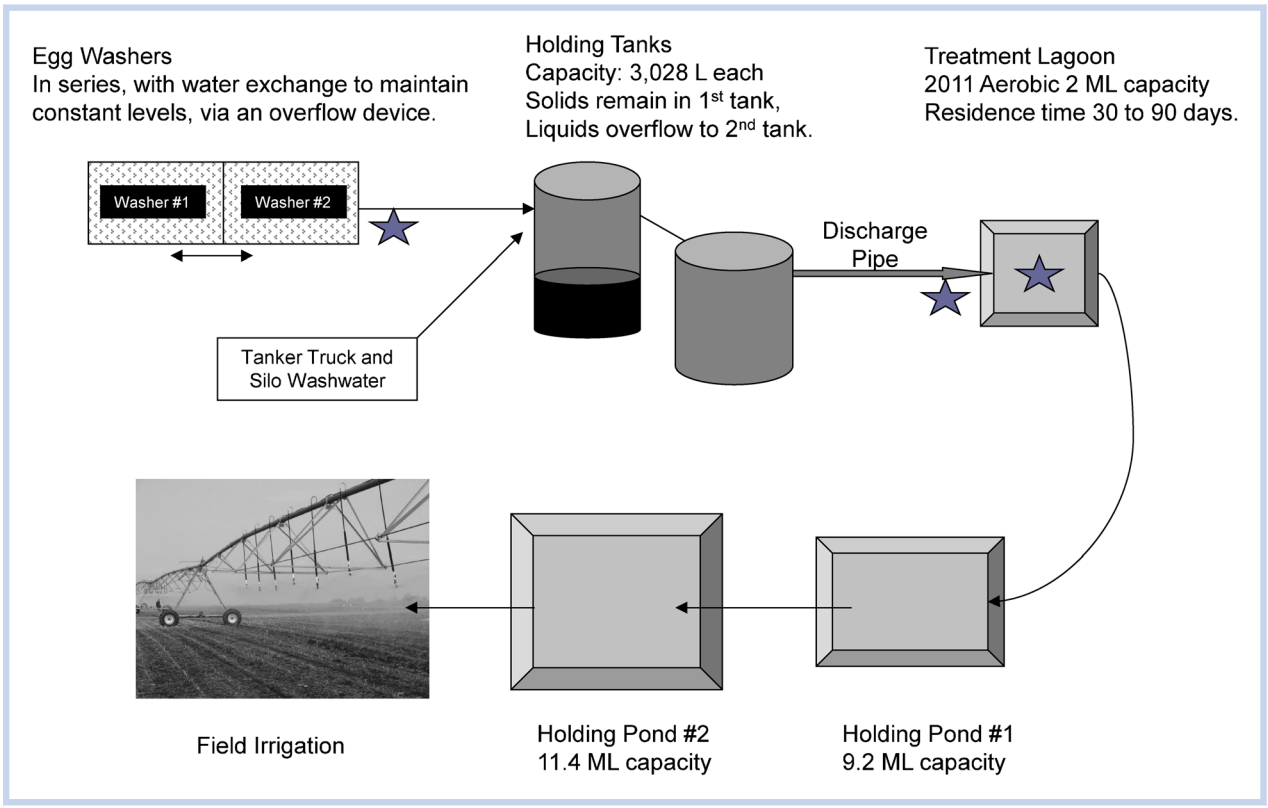


**Figure 3.** Schematic of 2011 washwater flow and sampling locations. Sampling location indicated by star. In 2010, the flow into lagoons was reversed, with holding pond 2 serving as the anaerobic treatment lagoon.

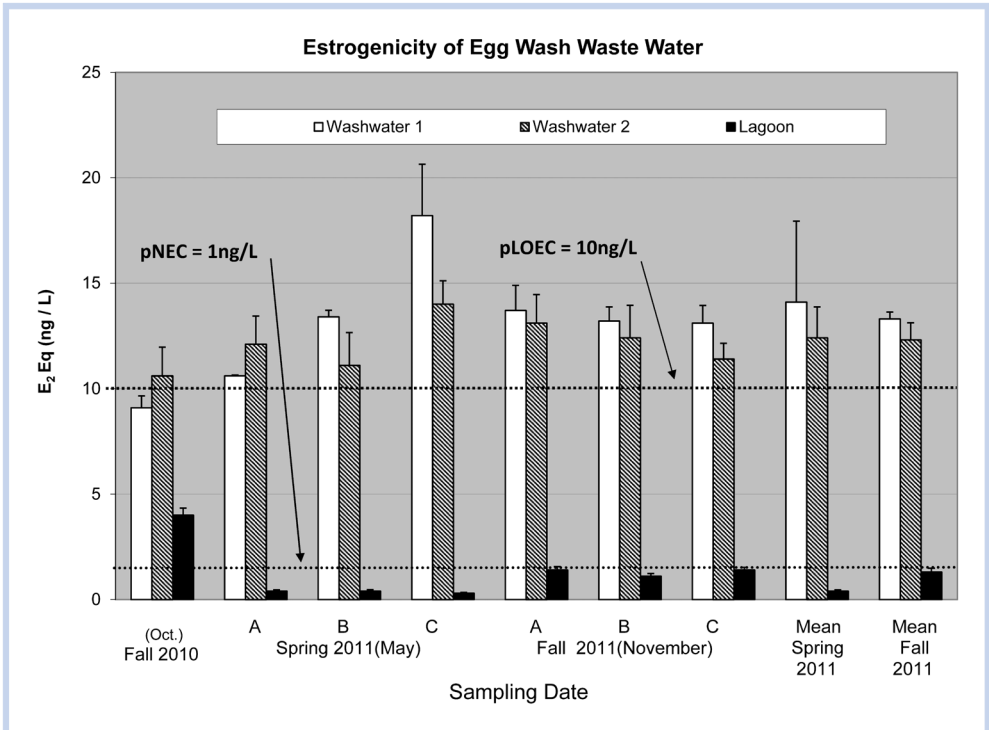


**Figure 4.** Estrogenicity of egg wash wastewater evaluated by E-Screen. A single set of samples was collected in the Fall (October) of 2010 from egg Washer 1 and 2 (operating in series), and a composite sample was collected from the anaerobic treatment lagoon. Values are mean ± standard deviation (SD) estradiol equivalents ($E_2$Eq, $n = 5$ wells per dilution of extract). After installation of an aeration system (February, 2011) followed by a period for stabilization of microflora, 2 sets of samples were collected on 3 consecutive days in the Spring (May) and Fall (November) of 2011, to determine if seasonal effects would occur on estrogenic activity. The mean of the Spring and Fall 2011 samples are presented in the last set of bars, with error bars reflecting the SDs of the 3 different collection days. Although there was no difference in $E_2$Eq from washwater from Washer 1 and 2 ($p = 0.32$), the $E_2$Eq of treatment lagoon was different than the washwater ($p < 0.0001$). No seasonal differences were found in the $E_2$Eq ($p = 0.97$). The proposed predicted no effect concentration (pNEC) of 17β-$E_2$ (Young et al. 2002) and proposed lowest observable effect concentrations (pLOEC) in fish (Nimrod et al. 1998) are indicated at 1 and 10 ng/L $E_2$Eq, respectively.

dextran-stripped fetal bovine serum). Egg washwater samples fell within the linear range of the E-Screen ($\approx 1 \times 10^{-12}$ to $1 \times 10^{-11}$ M on cells) when tested at 0.05 to 0.3× their original strength, whereas lagoon samples were typically within range when concentrated to 3-fold their original concentrations. Chemicals in use at the facility were tested at concentrations estimated to be present in the waste stream. To ensure sample extracts were not exerting toxic effect on the cells, proliferation was assessed in 1 well of each dilution of the sample extract fortified with $4 \times 10^{-12}$ M 17β-$E_2$. Toxicity was evident if proliferation in this well was less than proliferation in the presence of 17β-$E_2$ alone. The specificity of the proliferative response was verified as estrogen receptor-dependent through ablation with coincubations with $E_2$-receptor antagonist ICI 182,780 (Tocris, Ellisville, MO) as described by Rassmussen and Nielsen (2002).

### *Chemical analysis*

Aqueous sample extracts prepared for E-Screen were diluted with internal standards ($d_2$- estriol, $d_4$-estrone, $d_4$-17β-$E_2$, and $d_4$ EE2; final concentration of 20 pg/μL) for analysis by LC-$MS^2$ (as described in Shappell et al. 2007). When extracts were analyzed at approximately 700 times the original concentration, no deuterated internal standards (added at the point of extract dilution) were detectable. Therefore, a series of dilutions was made of an egg washwater sample with internal standards, to determine at what point the reduction in ion suppression and/or matrix interference allowed for detection of the deuterated internal standards. When extracts were approximately 31 times the original concentration, the peak areas of the deuterated internal standards were approximately 30% of those of the same standards added to the np$H_2O$ method extraction blanks. All samples were subsequently analyzed at the approximately 31× concentration, using a 20 μL injection volume.

### *Statistical analyses*

Data from 2011 were analyzed using a mixed-model analysis of variance, with season and sample source as fixed effects and sampling date as the blocking variable (SAS V9.3; SAS Institute, Cary, NC). Sample source differences were tested using Tukey's contrasts and seasonal differences using a priori contrasts.

## RESULTS AND DISCUSSION

### *Preliminary sample testing (Fall: October 2010)*

None of the chemicals used within the plant to clean equipment or process the eggs (that would end up in lagoon or egg wash wastewater) were estrogenic or toxic in the E-Screen assay at concentrations in use. Alkali 256, Durafoam 263, Chloroclean 296, Suppressor 3110, and Apex 292 were tested over a range of dilutions including those estimated to be present in the waste stream (1:1250, 1:2130, 1:533, 1:500, and 1:200, respectively, for commercial products).

The preliminary testing of the wastewater stream in Fall (October) 2010 indicated that $E_2$Eq of water from Washer 1 to be 9.1 ng/L (3 h of operation, ≈389 000 eggs); samples from Washer 2 were 0.83 ng/L (1 h, ≈130 000 eggs) and 10.58 ng/L (4 h, ≈518 000 eggs). Coefficients of variation for these samples averaged 8.5% ($n = 5$ wells per dilution). The concentrations of $E_2$Eq in raw egg washwater at the end of the 4-h wash cycle was therefore proximal to the proposed lowest observable effect concentration (pLOEC) for 17β-$E_2$ of 10 ng/L used by Young et al. (2002) based on larval 28-day exposure of Japanese Medaka producing only female fish (Nimrod and Benson 1998). Similarly, Seki et al. (2005) reported an LOEC of 8.66 ng/L for 17β-$E_2$ induction of vitellogenin in males, and decreased fertility of male-female pairs in 29-day exposures of the same fish species. The proposed predicted no effect concentration (pNEC) for $E_2$ was set at 1 or 2 ng/L, respectively (Young et al. 2002; Caldwell et al. 2012). A treatment lagoon inlet pipe grab sample had 3.49 ng/L $E_2$Eq, whereas a second grab sample taken 3.33 h later had so little activity that it only became apparent when tested at 2 times the original concentration and was still below the assay limits of quantitation (BLQ, 0.54 ng/L on plate or <0.15 ng/L in the original sample). The composite sample from the anaerobic treatment lagoon was very similar to the initial pipe grab sample, with 3.96 ng/L $E_2$Eq, or lower than the pLOEC (Young et al. 2002). Concentration of $E_2$Eq in the subsequent storage ponds could reasonably be expected to be well below the limits of quantitation, as typically estrogens are further degraded in 2° and 3° storage ponds. For example, Hutchins et al. (2007) reported estrogen concentrations decreased 2 orders of magnitude from 1° to 3° poultry lagoons of laying hen waste, as well as similar reductions in swine and dairy waste lagoons. Based on these findings, plans to assay estrogenicity of storage ponds and runoff postaerial irrigation were abandoned.

### *Spring (May) 2011 testing*

Despite consistent timing of sampling in May of 2011 consistent with the Fall (October) 2010 sampling period (egg washwater from Washer 1 and 2 after 4 h of use, grab samples from the inlet pipe collected ≈1.5 h later, and treatment lagoon samples collected 5 min later, all on 3 consecutive days) the same variability was measured in the grab samples from the inlet pipe as previously found (5/16 and 5/18 samples were BLQ; whereas the 5/17 sample had 4.4 ng/L $E_2$Eq with a coefficient of variation [COV] of 13%). When plant personnel were questioned about potential explanations for sporadic elevated pipe grab samples, they mentioned the practice of "tanker washout." This occurred when refrigerated tankers arrived at the plant with residue from other products, typically milk or cream, that needed to be washed out before loading with egg product. The plant allowed tankers to use their water supply and the wash water would empty into serial holding tanks. In addition, rinse water from silos containing raw egg product also emptied into these holding tanks. Once the level of the holding tank was sufficiently elevated, it released the upper most liquid into the treatment lagoon inlet pipe. If the tanker had contained cream, this would be expected to float to the top of the holding tank and be the first to be released. Milk is known to contain 17β-$E_2$ and it is present at even higher concentrations in the fat-lipid phase (≈50% higher at ≈35 ng/L) (Tso and Aga 2010). Because of this variability in pipe grab samples, the sampling protocol was modified in the last collection period to include only composite samples from the treatment lagoon.

The estrogenic activity of egg Washers 1 and 2 were similar for all 3 days tested (Figure 3) ranging from 10.6 to 18.2 ng/L $E_2$Eq. The estrogenic activity of the composite treatment lagoon samples was extremely consistent from day to day (0.3 to 0.4 ng/L), as would be expected. This data set indicated the relative daily consistency of the $E_2$Eq produced from a 4-h egg wash cycle, with concentrations of raw wastewater being somewhat above the pLOEC. The $E_2$Eq of wastewater from

the treatment lagoon fell below the pNEC of 1 or 2 ng/L for estradiol in fish (Young et al. 2002; Caldwell et al. 2012). It was unclear if the drop in $E_2$Eq in wastewater from the treatment lagoon, relative to the October 2010 sample, was a seasonal effect, due to higher lagoon temperatures in May versus November, and therefore increased microbial degradation (Zheng et al. 2012), or related to the conversion of the treatment lagoon from an anaerobic to aerobic system. A comparison of average daily ambient air temperatures for the 2 weeks immediately before the sampling period were nearly identical (14.4° C and 15.0° C for October 2010 and May 2011, respectively, Columbus, OH, ≈50 km away) (NOAA 2010-2011). Data from later samples collected in November of the same year point to the conversion to an aerobic treatment system, as supported by literature reports (Fan et al. 2007). Change in treatment lagoon capacity (from $11.4 \times 10^6$ L for the anaerobic 2010 system to $2 \times 10^6$ L for the aerobic 2011 system) might have resulted in an increase in estrogenicity, as daily inputs would be less diluted in the 80% smaller treatment lagoon of 2011.

### *Fall (November) 2011 testing*

Estrogenic activity over the 3 day collection for Fall (November) 2011 samples was even more consistent (Figure 3) than data from the previous collection events. Estrogenic activity of egg wash water ranged from 11.4 to 13.7 ng/L $E_2$Eq whereas the COVs across 3 days were 2%, 7%, and 13% for egg Washer 1, 2, and treatment lagoon composite, respectively. The higher COV measured in the treatment lagoon samples is a reflection of the lower estrogenic activity concentrations, as daily values were 1.4, 1.0, and 1.4 ng/L. Although no seasonal effect ($p > 0.05$) was documented across sample type, the consistencies of the composite lagoon values may indicate an ever so slightly higher $E_2$Eq in Fall (November) samples than Spring (May) (0.37 and 1.3 ng/L). It would be hard to argue such a minimal increase in $E_2$Eq concentration would result in a difference in organismal response to such an exposure. Daily average ambient air temperature for the Fall 2011 period was 6.1° C in contrast to the Spring 2011 average of 15.0° C. With a 10° C increase in temperature from 15 to 25° C, Zheng et al. (2012) reported a near doubling of degradation rates of 17β-$E_2$ in dairy lagoon waste under anaerobic conditions, so a proportional increase might occur under aerobic conditions from 6 to 15° C. Although the treatment lagoon was not sampled during the summer heat, concentrations of $E_2$Eqs could be expected to fall even lower than those measured in the Spring (May) of 2011. It is clear that the treatment lagoon concentrations were consistently in the pNEC range, and therefore lagoon water should not pose a threat of estrogenic endocrine disruption when land applied.

It was not possible to quantify the estrogens in extracted samples by LC $MS^2$. All samples were analyzed by LC-$MS^2$, but matrix-effects at higher extract concentrations resulted in ion suppression to such an extent that internal standards (IS) were not detectable in an acceptable range. Dilutions that provided peak areas of the deuterated internal standards added at the time of dilution that were approximately 30% of those in the np$H_2O$ extraction blanks, resulted in maximal estrone or 17β-$E_2$ peaks of ≤5% of the peak areas of the concomitant IS (20 pg/μL). Although the parent ions of estrone and 17β-$E_2$ ions were present in some egg washwater extracts when analyzed by LC-$MS^2$ at 31 times environmental concentrations, confirmatory fragments were not detectable. Similar ion suppression was found with lagoon extracts. The limit of quantitation (LOQ) for estrone and 17β-$E_2$ are 2 pg/μL on column in our laboratory, whereas the E-Screen LOQ on plate is 0.54 fg/μL of $E_2$Eq on plate (a 350 times lower LOQ than the $MS^2$). The highest concentration of extract tested on cells was approximately 3 times the environmental concentration, which would translate to detection of 0.15 fg/μL or 0.15 ng/L. Because of matrix interference, the highest concentration of sample that could be analyzed by LC-$MS^2$ was 31-fold environmental, so the LOQ in sample would be equivalent to 0.06 pg/μL or 60 ng/L. Under these conditions, if all the $E_2$Eq detected by E-Screen were the result of 17β-$E_2$, even in the case of the highest $E_2$Eq of approximately 18 ng/L, quantitation would not be possible. If the $E_2$Eq were the result of estrone, which has 1/100th of the relative $E_2$Eq of 17β-$E_2$ (Soto et al. 1995), 18 ng/L could translate into 1800 ng/L of estrone, which should be detectable based on our LC-$MS^2$ LOQ. Because of matrix interference, this was not the case.

Although steroid hormones in hens are known to be age- and molt status-dependent, and environmental cues such as photoperiod influence sexual maturity and onset of egg laying (Etches 1996), the egg industry has refined its management system to minimize the effect of these factors on egg production. At this particular facility, laying hens are staggered by age from 20 to 110 weeks by approximately 3-month intervals, in 8 separate houses. Birds just coming into egg production are exposed to an increasing light regimen (from 12 to 15 h of light, 1 house only) whereas the other houses with the exception of the one cycling through molt, are on a constant light-dark cycle of 12:12. With this type of "staging" the egg supply is essentially constant through out the year, providing approximately 450 million kg of egg product per week. An assumption is made that if facility production can be maintained essentially constant through these management practices, then the inputs from the birds, on a whole facility basis, would be relatively constant, and therefore the total estrogenic activity found in egg washwater would remain relatively constant.

### *Implications*

Based on relative concentrations of 17β-$E_2$, the source of estrogenic activity in the egg washwater could likely be manure removed from the outside of the eggs versus the egg material itself. Although analysis for 17β-$E_2$ by gas chromatography tandem mass spectrometer (GC-$MS^2$) has not been reported specifically for layer manure, maximum concentrations by radio-immuno assay were approximately 60 ng/g wet weight (Shore et al. 1993). The concentration of 17β-$E_2$ in eggs was reported as 1.45 ng/g egg weight (GC-$MS^2$ analysis) (Courant et al. 2007), or less than 40 times that in manure. Although egg breakage does occur in the wash process, breakage is typically minimal, and therefore manure could be expected to be the primary source of estrogenic activity in egg wash wastewater. Although estrogenic activity in the treatment lagoon may have resulted in part from the silo washwater (diluted raw egg product), its contribution would be consistent week to week.

Land application of lagoon waste, independent of animal source, is dependent on the nutrient needs of the soil. Typical application rates of chemical or natural manures are 50 lb/acre (9.2 kg/ha) of inorganic phosphate, and 175–200 lb/acre (32–37 kg/ha) for N. Averages for egg wash lagoon wastewater in 2011 were 50 ppm N and 35 ppm phosphate. At these concentrations and suggested application rates, the N load

would be accomplished by the application of $3.1 \times 10^6$ L/ha, but would be phosphate limited to $0.9 \times 10^6$ L/ha, or less than 1/10th of the terminal storage lagoon's capacity. If the estrogenic activity of egg wash wastewater were to fall 2 orders of magnitude in storage ponds, as indicated by the data of Hutchins et al. (2007), the resultant concentrations would be projected to be $\leq$0.013 ng/L $E_2$Eq. For context, Caldwell et al. (2010) predicted environmental concentrations of 17β-$E_2$ in 90% of US drinking water to range from 0.02 (average flow conditions) to 0.19 ng/L (low flow conditions), with an average mean flow concentrations of 0.01 ng/L and low flow 0.05 ng/L. Even a 10% reduction in estrogenic activity of egg wash wastewater would yield concentrations equivalent to drinking water concentrations of 17β-$E_2$ under low flow conditions. Using models, these authors projected that human consumption of drinking water with these concentrations would result in 28 times less than the acceptable daily intake developed for sensitive populations. Because of the low concentrations of $E_2$Eq, N and P in egg wash wastewater, its land application serves the purpose of irrigation, increasing crop production, while sparing the use of groundwater. When considering approximately 74 000 L of water are used in a typical day of production at this facility, a rationale for conservation of water resources becomes obvious.

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## REFERENCES

Baird DT, Guevara A. 1969. Concentration of unconjugated estrone and estradiol in peripheral plasma in nonpregnant women throughout the menstrual cycle, castrate and postmenopausal women and in men. *J Clin Endocrinol* 29: 149–156.

Caldwell DJ, Mastrocco F, Nowak E, Johnston J, Yekel H, Pfeiffer D, Hoyt M, DuPlessie BM, Anderson PD. 2010. An assessment of potential exposure and risk from estrogens in drinking water. *Environ Health Perspect* 188:338–344.

Caldwell DJ, Mastrocco F, Anderson PD, Länge R, Sumpter JP. 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. *Environ Toxicol Chem* 31:1396–1406.

Cockrem JF, Rounce JR. 1994. Faecal measurements of oestradiol and testosterone allow the non-invasive estimation of plasma steroid concentrations in the domestic fowl. *Br Poult Sci* 35:433–443.

Courant F, Antignac JP, Maume D, Monteau F, Andre F, Le Bizec B. 2007. Determination of naturally occurring oestrogens and androgens in retail samples of milk and eggs. *Food Add Contam* 24:1358–1366.

Etches RJ. 1996. Growth and sexual maturation. In: Reproduction in poultry. Cambridge, UK: CAB International. p 96, 268, 288.

Fan Z, Casey FXM, Hakk H, Larsen GL. 2007. Persistence and fate of 17β-estradiol and testosterone in agricultural soils. *Chemosphere* 67:886–895.

Hutchins SR, White MR, Hudson FM, Fine DD. 2007. Analysis of lagoon samples from different concentrated animals feeding operations for estrogens and estrogen conjugates. *Environ Sci Technol* 41:738–744.

Munro CJ, Stabenfeldt GH, Cragun JR, Addiego LA, Overstreet JW, Lasley BL. 1991. Relationship of serum estradiol and progesterone concentrations to the excretion profiles of their major urinary metabolites as measured by enzyme immunoassay and radioimmunoassay. *Clin Chem* 37:838–844.

Nimrod AC, Benson WH. 1998. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. *Aquatic Toxicol* 44:141–156.

[NOAA] National Oceanic and Atmospheric Administration. 2010–2011. Preliminary local climatological data. National Weather Service for Columbus, Ohio. [cited 2013 April 16]. Available from: http://www.nws.noaa.gov/climate/index.php?wfo=iln

Rassmussen TH, Nielsen JB. 2002. Critical parameters in the MCF-7 cell proliferation bioassay (E-screen). *Biomarkers* 7:322–336.

Seki M, Yokota H, Maeda M, Kobayashi K. 2005. Fish full life-cycle testing for 17 beta-estradiol on medaka (Oryzias latipes). *Environ Toxicol Chem* 24:1259–1266.

Shappell NW. 2006. Estrogenic activity in the environment: Municipal wastewater effluent, river, ponds, and wetlands. *J Environ Qual* 35:122–132.

Shappell NW, Billey LO, Forbes D, Matheny TA, Poach ME, Reddy GB, Hunt PG. 2007. Estrogenic activity and steroid hormones in swine wastewater through a lagoon constructed wetland system. *Environ Sci Technol* 41:444–450.

Shappell NW, Mostrom MS, Lenneman EM. 2012. E-Screen evaluation of sugar beet feedstuffs in a case of reduced embryo transfer efficiencies in cattle: the role of phytoestrogens and zearalenone. *In Vitro Cell Dev Biol Animal* 48:216–228.

Shore LS, Gurevitz M, Shemesh M. 1993. Estrogen as an environmental pollutant. *Bull Environ Contam Toxicol* 51:361–366.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. 1995. The E-screen assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ Health Perspect* 103(Suppl7): 113–122.

Tso J, Aga DS. 2010. A systematic investigation to optimize simultaneous extraction and liquid chromatography tandem mass spectrometry analysis of estrogens and their conjugated metabolites in milk. *J Chromatogr* 1217:4784–4795.

Young WF, Whitehouse P, Johnson I, Sorokin N. 2002. Proposed predicted no effect concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. Environment Agency. R&D technical report P2-T04/1. Bristol, UK: England and Wales Environment Agency. p 93–95.

Zheng W, Li X, Yates SR, Bradford SA. 2012. Anaerobic transformation kinetics and mechanism of steroid estrogenic hormones in dairy lagoon water. *Environ Sci Technol* 46:5471–5478.